



MAQC Study Affymetrix Platform Data Generation Plan

Test Sites

1. **Test site:** Emerging Markets and Molecular Diagnostics R & D,
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2. **Test site:** Food and Drug Administration
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3. **Test site:** Ambion, Inc.
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4. **Test site:** David Geffen School of Medicine, UCLA
 Contact Person: Charles Wang
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5. **Test site:** EPA
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Affymetrix. MAQC.SOP

1. Introduction

The purpose of this procedure is standardization of protocols used by MAQC test sites using the Affymetrix GeneChip® platform.

2. Materials

Starting Material

5µg of total RNA. From 5µg of high quality total RNA, ~150 to 200µg of cRNA will be produced. cRNA yield and quality will be evaluated using the bioanalyzer with an average peak height of 1500nt.

Equipment

ITEM	1° Source
Affymetrix GeneChip® Operating System	GCOS V.n*
Heat Block or Thermal Cycler	*n
0.5ml or 0.2ml Microfuge tubes	*n
1.5ml clear and amber tubes	*n
Pipettes and tips	*n
Microcentrifuge	*n
0.2µm filters	*n
Tough spots	USA Scientific #9185-0000
Agilent 2100 Bioanalyzer	*n
Nanodrop ND1000 Spectrophotometer	NanoDrop Technologies
GeneChip®Hybridization Oven 640	Affymetrix P/N 8001318
GeneChip®Fluidics Station 450 or 400	Affymetrix P/N 00-0079
GeneChip®Scanner 3000	Affymetrix P/N 00-0073

*n = site specific

Reagents

ITEM	1° Source
Affymetrix One-Cycle cDNA kit	Provided by Affymetrix
Poly A Control kit	Provided by Affymetrix
Affymetrix IVT kit	Provided by Affymetrix
Eukaryotic Hyb Control kit	Provided by Affymetrix
HG-U133 Plus 2.0 Arrays	Provided by Affymetrix
20 RNA samples, 5µg each	Provided by Ambion
Molecular biology-grade Water	*n
100% Ethanol	*n
Acetylated BSA	*n
Herring Sperm DNA (10mg/ml)	Promega #D1811
5M NaCl	Ambion #9760G
MES hydrate	Sigma #M5287
MES Sodium Salt	Sigma #M5057
0.5M EDTA	Sigma #E7889
DMSO	Sigma #D5879
10% Tween-20	Pierce #28320
20X SSPE	*n
R-Phycoerythrin Streptavidin	Molecular Probes#S-866
Goat IgG	Sigma #I5256
Biotinylated anti-streptavidin antibody	Vector Laboratories #BA-0500
RNA 6000 Nano Assay kit and chips	Agilent # 5065-4475 (assay) 5065-4476 (chips)
RNA 6000 Ladder	Ambion #7152

12X MES Stock Buffer

(1.22M MES, 0.89M [Na⁺])

For 1,000 mL:

64.61g of MES hydrate

193.3g of MES Sodium Salt

800 mL of Molecular Biology Grade water

Mix and adjust volume to 1,000 mL.

The pH should be between 6.5 and 6.7. Filter through a 0.2 µm filter.

2X Hybridization Buffer

(Final 1X concentration is 100 mM MES, 1M [Na⁺], 20 mM EDTA, 0.01% Tween-20)

For 50 mL:

8.3 mL of 12X MES Stock Buffer

17.7 mL of 5M NaCl

4.0 mL of 0.5M EDTA

0.1 mL of 10% Tween-20

19.9 mL of water

Store at 2°C to 8°C, and shield from light

Do not autoclave. Store at 2°C to 8°C, and shield from light. Discard solution if it oxidizes, turns yellow.

Wash Buffer A: Non-Stringent Wash Buffer

(6X SSPE, 0.01% Tween-20)

For 1,000 mL:

300 mL of 20X SSPE

1.0 mL of 10% Tween-20

699 mL of water

Filter through a 0.2 µm filter

Wash Buffer B: Stringent Wash Buffer

(100 mM MES, 0.1M [Na⁺], 0.01% Tween-20)

For 1,000 mL:

83.3 mL of 12X MES Stock Buffer

5.2 mL of 5M NaCl

1.0 mL of 10% Tween-20

910.5 mL of water

Filter through a 0.2 µm filter

Store at 2°C to 8°C and shield from light

2X Stain Buffer

(Final 1X concentration: 100 mM MES, 1M [Na⁺], 0.05% Tween-20)

For 250 mL:

41.7 mL of 12X MES Stock Buffer

92.5 mL of 5M NaCl

2.5 mL of 10% Tween-20

113.3 mL of water

Filter through a 0.2 µm filter

Store at 2°C to 8°C and shield from light

10 mg/mL Goat IgG Stock

Resuspend 50 mg in 5 mL of 150 mM NaCl

Store at 4°C

If a larger volume of the 10 mg/mL IgG stock is prepared, aliquot and store at -20°C until use. After the solution has been thawed it should be stored at 4°C. Avoid additional freezing and thawing.

3. RNA Preparation

- 3.1 Each site will be provided 4 tubes of stock RNA, representing Stratagene Universal Human Reference RNA, Ambion Brain Reference RNA, and two titration mixtures of these RNA.
- 3.2 RNA quantity assessment should be done on NanoDrop by taking 1µl of each RNA sample from the tube of stock RNA.
- 3.3 RNA quality assessment on Agilent Bioanalyzer should be done on each stock tube prior to start of procedure by running 200ng of each total RNA on the Nano Chip.
- 3.4 Save raw data files from Bioanalyzer assay and record 28S/18S ratio and RIN, if available.
- 3.5 5 aliquots of 5µg each should be prepared from each stock RNA tube for a total of 20 tubes.

4. Procedures for the Sample Preparation and Array Hybridization from 5µg Total RNA

4.1 One-Cycle cDNA Synthesis and Clean up

Step 1: Preparation of Poly-A RNA Controls for One-Cycle cDNA Synthesis (Spike-in Controls)

(Eukaryotic Poly-A RNA Control Kit is used for this step)

Designed specifically to provide exogenous positive controls to monitor the entire eukaryotic target labeling process, a set of poly-A RNA controls is supplied in the GeneChip® Eukaryotic Poly-A RNA Control Kit.

Each eukaryotic GeneChip® probe array contains probe sets for several *B. subtilis* genes that are absent in eukaryotic samples (*lys*, *phe*, *thr*, and *dap*). These poly-A RNA controls are *in vitro* synthesized, and the polyadenylated transcripts for the *B. subtilis* genes are premixed at staggered concentrations. The concentrated Poly-A Control Stock can be diluted with the Poly-A Control Dil Buffer and spiked directly into RNA samples to achieve the final concentrations (referred to as a ratio of copy number) summarized below.

The controls are then amplified and labeled together with the samples. Examining the hybridization intensities of these controls on GeneChip® arrays help to monitor the labeling process independently from the quality of the starting RNA samples.

Final Concentrations of Poly-A RNA Controls in Samples: *lys* 1:100,000 ;*phe* 1:50,000 ;*thr* 1:25,000 ;*dap* 1:6,667

The Poly-A RNA Control Stock and Poly-A Control Dil Buffer are provided with the kit to prepare the appropriate serial dilutions.

To prepare the poly-A RNA dilutions for 5 µg of total RNA:

1. Add 2 µL of the Poly-A Control Stock to 38 µL of Poly-A Control Dil Buffer for the First Dilution (1:20).
2. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
3. Add 2 µL of the First Dilution to 98 µL of Poly-A Control Dil Buffer to prepare the Second Dilution (1:50).
4. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
5. Add 5µL of the Second Dilution to 45 µL of Poly-A Control Dil Buffer to prepare the Third Dilution (1:10).
6. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
7. Add 2 µL of this Third Dilution to 5 µg of sample total RNA.

Step 2: First-Strand cDNA Synthesis
(One-Cycle cDNA Synthesis Kit is used for this step)

Notes:

- a) Briefly spin down all tubes in the Kit before using the reagents.
- b) Perform all of the incubations in thermal cyclers with the heated lid off. The following program can be used as a reference to perform the first-strand cDNA synthesis reaction in a thermal cycler; the 4°C holds are for reagent addition steps:
70°C 10 minutes
4°C hold
42°C 1 hour
4°C hold

1. Mix RNA sample, diluted poly-A RNA controls, and T7-Oligo(dT) Primer.

RNA/T7-Oligo(dT) Primer Mix Preparation for 5 µg of total RNA.

Component	Volume (ul)
Sample RNA	Variable/ 5µg
Diluted poly-A RNA Controls	2.0
T7-Oligo(dT) Primer, 50 µM	2.0
RNase-free Water	Variable
Total Volume	12.0

- 1.1 Place total RNA (5µg) in a PCR tube.
- 1.2 Add 2 µL of the appropriately diluted poly-A RNA controls (See Step 1: Preparation of Poly-A RNA Controls for One-Cycle cDNA Synthesis (Spike-in Controls)).
- 1.3 Add 2 µL of 50 µM T7-Oligo(dT) Primer.
- 1.4 Add RNase-free Water to a final volume of 12 µL .
- 1.5 Gently flick the tube a few times to mix, and then centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube.
- 1.6 Incubate the reaction for 10 minutes at 70°C.
- 1.7 Cool the sample at 4°C for at least 2 minutes.
- 1.8 Centrifuge the tube briefly (~5 seconds) to collect the sample at the bottom of the tube.

2. In a separate tube, assemble the First-Strand Master Mix.

Component	Per Sample (ul)	X22 (ul)
5X First Strand Reaction Mix	4.0	88.0
DTT, 0.1M	2.0	44.0
dNTPs, 10mM	1.0	22.0
SuperScript II	1.0	22.0
Total Volume	8.0	176.0

Note: Fully suspend the master mix before adding to the samples as DTT may form precipitation.

- 2.1 Prepare sufficient First-Strand Master Mix for all of the RNA samples. When there are more than 2 samples, it is prudent to include additional material to compensate for potential pipetting inaccuracy or solution lost during the process. The above recipe with 10% surplus taken into account:
- 2.2 Aliquot 8 μ l of the master mix to the 12 μ l RNA-primer mix
- 2.3 Mix thoroughly and quick spin samples
- 2.4 Incubate at 42°C for 1 hour
- 2.5 Cool the samples for at least 2 minutes at 4 °C but not longer than 10 minutes.

Note: Cooling the samples at 4°C is required before proceeding to the next step. Adding the Second-Strand Master Mix directly to solutions that are at 42°C will compromise enzyme activity.

After incubation at 4°C, centrifuge the tube briefly (~5 seconds) to collect the reaction at the bottom of the tube and immediately proceed to *Step 3: Second-Strand cDNA*

Step 3: Second-Strand cDNA Synthesis and Clean up
(One-Cycle cDNA Synthesis Kit is used for this step)

1. In a separate tube, assemble Second-Strand Master Mix.

- 1.1 Prepare sufficient Second-Strand Master Mix using the following recipe.

Component	Per Sample (ul)	X22 (ul)
H2O (RNase- free)	91.0	2002.0
5X 2nd Strand Reaction Mix	30.0	660.0
dNTP, 10mM	3.0	66.0
E.coli DNA Ligase	1.0	22.0
E.coli DNA Polymerase I	4.0	88.0
RNase H	1.0	22.0
Total Volume	130.0	2860.0

- 1.2 Mix well by gently flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the solution at the bottom of the tube.
2. Add 130 µL of Second-Strand Master Mix to each first-strand synthesis sample from *Step 2: First-Strand cDNA Synthesis* for a total volume of 150 µL. Gently flick the tube a few times to mix, and then centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube.
3. Incubate for 2 hours at 16°C.
4. Add 2 µL of T4 DNA Polymerase to each sample and incubate for 5 minutes at 16°C.
5. After incubation with T4 DNA Polymerase add 10 µL of EDTA, 0.5M and proceed to

Cleanup of Double-Stranded cDNA using Affymetrix Sample Cleanup Module

BEFORE STARTING, please note:

- *cDNA Wash Buffer is supplied as a concentrate. Before using for the first time, add 24 mL of ethanol (96-100%), as indicated on the bottle, to obtain a working solution, and checkmark the box on the left-hand side of the bottle label to avoid confusion.*
- *All steps of the protocol should be performed at room temperature. During the procedure, work without interruption.*
- *If cDNA synthesis was performed in a reaction tube smaller than 1.5 mL, transfer the reaction mixture into a 1.5 or 2 mL microfuge tube (not supplied) prior to addition of cDNA Binding Buffer.*

1. Add 600 μ L of cDNA Binding Buffer to the double-stranded cDNA synthesis preparation. Mix by vortexing for 3 seconds.
2. Check that the color of the mixture is yellow (similar to cDNA Binding Buffer without the cDNA synthesis reaction). *(If the color of the mixture is orange or violet, add 10 μ L of 3M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.)*
3. Apply 762 μ L of the sample to the cDNA Cleanup Spin Column sitting in a 2 mL Collection Tube (supplied), and centrifuge for 1 minute at $\geq 8,000 \times g$ ($\geq 10,000$ rpm). Discard flow-through.
4. Transfer spin column into a new 2 mL Collection Tube (supplied). Pipet 750 μ L of the cDNA Wash Buffer onto the spin column. Centrifuge for 1 minute at $\geq 8,000 \times g$ ($\geq 10,000$ rpm). Discard flow-through.
5. Open the cap of the spin column and centrifuge for 5 minutes at maximum speed ($\leq 25,000 \times g$). Discard flow-through and Collection Tube. Place columns into the centrifuge using every second bucket. Position caps over the adjoining bucket so that they are oriented in the opposite direction to the rotation (i.e., if the microcentrifuge rotates in a clockwise direction, orient the caps in a counterclockwise direction). This avoids damage of the caps. Centrifugation with open caps allows complete drying of the membrane.
6. Transfer spin column into a 1.5 mL Collection Tube, and pipet 22 μ L of cDNA Elution Buffer directly onto the spin column membrane. Incubate for 1 minute at room temperature and centrifuge 1 minute at maximum speed ($\leq 25,000 \times g$) to elute. Ensure that the cDNA Elution Buffer is dispensed directly onto the membrane. The average volume of eluate is 20 μ L from 22 μ L Elution Buffer.
7. After cleanup, please proceed to *Synthesis of Biotin-Labeled cRNA*.

4.2 Synthesis of Biotin-Labeled cRNA

Store all reagents in a -20°C freezer that is not self-defrosting. Prior to use, centrifuge all reagents briefly to ensure that the solution is collected at the bottom of the tube. The Target Hybridizations and Array Washing protocols have been optimized specifically for this IVT Labeling Protocol. Closely follow the recommendations described below for maximum array performance.

1. Use the following table to prepare master mix for the IVT reactions:

Component	Per Sample (ul)	X22 (ul)
10X IVT Labeling Buffer	4.0	88.0
IVT Labeling NTP Mix	12.0	264.0
IVT Labeling Enzyme Mix	4.0	88.0
Total Volume	20.0	440.0

Note: Do not assemble the reaction on ice, since spermidine in the 10XIVT labeling buffer can lead to precipitation of the template cDNA.

2. Aliquot 20ul of the master mix to the 20ul cleaned-up cDNA.
3. Carefully mix the reagents and collect the mixture at the bottom of the tube by brief (~5 seconds) microcentrifugation.
4. Incubate at 37°C for 16 hours. To prevent condensation, incubations are best performed in a thermal cycler.
5. Store labeled cRNA at -20°C, or -70°C if not purifying immediately. Alternatively, proceed to *Cleanup and Quantification of Biotin-Labeled cRNA*.

4.3 Cleanup and Quantification of Biotin-Labeled cRNA

(Sample Cleanup Module is used for cleaning up the biotin-labeled cRNA)

Step 1: Cleanup of Biotin-Labeled cRNA

It is essential to remove unincorporated NTPs, so that the concentration and purity of cRNA can be accurately determined by 260 nm absorbance.

- *DO NOT extract biotin-labeled RNA with phenol-chloroform. The biotin will cause some of the RNA to partition into the organic phase. This will result in low yields.*
 - *Save an aliquot of the unpurified IVT product for analysis by gel electrophoresis.*
 - *IVT cRNA Wash Buffer is supplied as a concentrate. Before using for the first time, add 20 mL of ethanol (96-100%), as indicated on the bottle, to obtain a working solution, and checkmark the box on the left-hand side of the bottle label to avoid confusion.*
 - *IVT cRNA Binding Buffer may form a precipitate upon storage. If necessary, redissolve by warming in a water bath at 30°C, and then place the buffer at room temperature.*
 - *All steps of the protocol should be performed at room temperature. During the procedure, work without interruption.*
1. Add 60 µL of RNase-free Water to the IVT reaction and mix by vortexing for 3 seconds.
 2. Add 350 µL IVT cRNA Binding Buffer to the sample and mix by vortexing for 3 seconds.
 3. Add 250 µL ethanol (96-100%) to the lysate, and mix well by pipetting. Do not centrifuge.
 4. Apply sample (700 µL) to the IVT cRNA Cleanup Spin Column sitting in a 2 mL Collection Tube. Centrifuge for 15 seconds at $\geq 8,000 \times g$ ($\geq 10,000$ rpm). Discard flow-through and Collection Tube.
 5. Transfer the spin column into a new 2 mL Collection Tube (supplied). Pipet 500 µL IVT cRNA Wash Buffer onto the spin column. Centrifuge for 15 seconds at $\geq 8,000 \times g$ ($\geq 10,000$ rpm) to wash. Discard flow-through.
 6. Pipet 500 µL 80% (v/v) ethanol onto the spin column and centrifuge for 15 seconds at $\geq 8,000 \times g$ ($\geq 10,000$ rpm). Discard flow-through.
 7. Open the cap of the spin column and centrifuge for 5 minutes at maximum speed ($\leq 25,000 \times g$). Discard flow-through and Collection Tube. Place columns into the centrifuge using every second bucket. Position caps over the adjoining bucket so that they are oriented in the opposite direction to the rotation (i.e., if the microcentrifuge rotates in a clockwise direction, orient the caps in a counterclockwise direction). This avoids damage of the caps. Centrifugation with open caps allows complete drying of the membrane.
 8. Transfer spin column into a new 1.5 mL Collection Tube (supplied), and pipet 100 µL of RNase-free Water directly onto the spin column membrane. Ensure that the water

is dispensed directly onto the membrane. Centrifuge 1 minute at maximum speed ($\leq 25,000 \times g$) to elute.

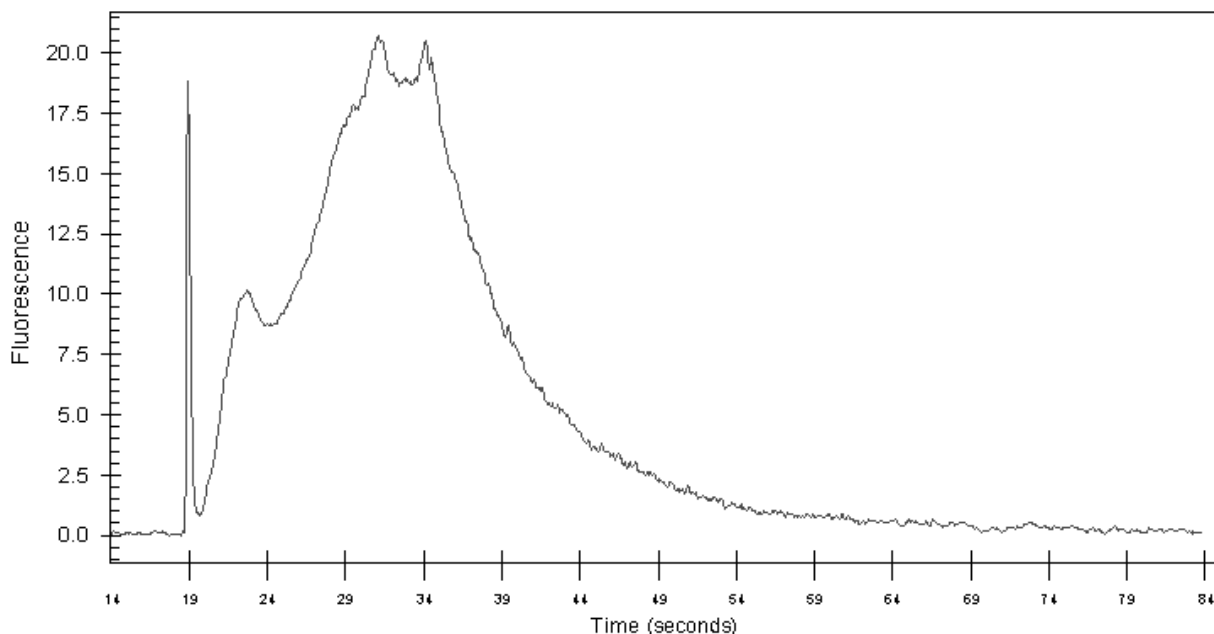
9. Store cRNA at -20°C , or -70°C if not quantitated immediately. Alternatively, proceed to *Step 2: Quantification of the cRNA*.

Step 2: Quantification of the cRNA

1. Aliquot 1ul of purified cRNA and measure the concentration using NanoDrop. Save the quantitation results as an Excel file and calculate and total cRNA yield from 5 μg total RNA.
 - 1.1 Check the absorbance at 260 nm and 280 nm to determine sample concentration and purity.
 - 1.2 Maintain the A260/A280 ratio close to 2.0 for pure RNA (ratios between 1.9 and 2.1 are acceptable).

Step 3: Checking Unfragmented Samples by Gel Electrophoresis

Use 200ng of cRNA to run Bioanalyzer (mRNA Smear) using RNA Nano 6000 chips to estimate the yield and size distribution of labeled transcripts. The following are examples of typical cRNA products examined on an Agilent 2100 Bioanalyzer. The cRNAs have a size range from 500nt to 2000nt with a peak around 1500nt.

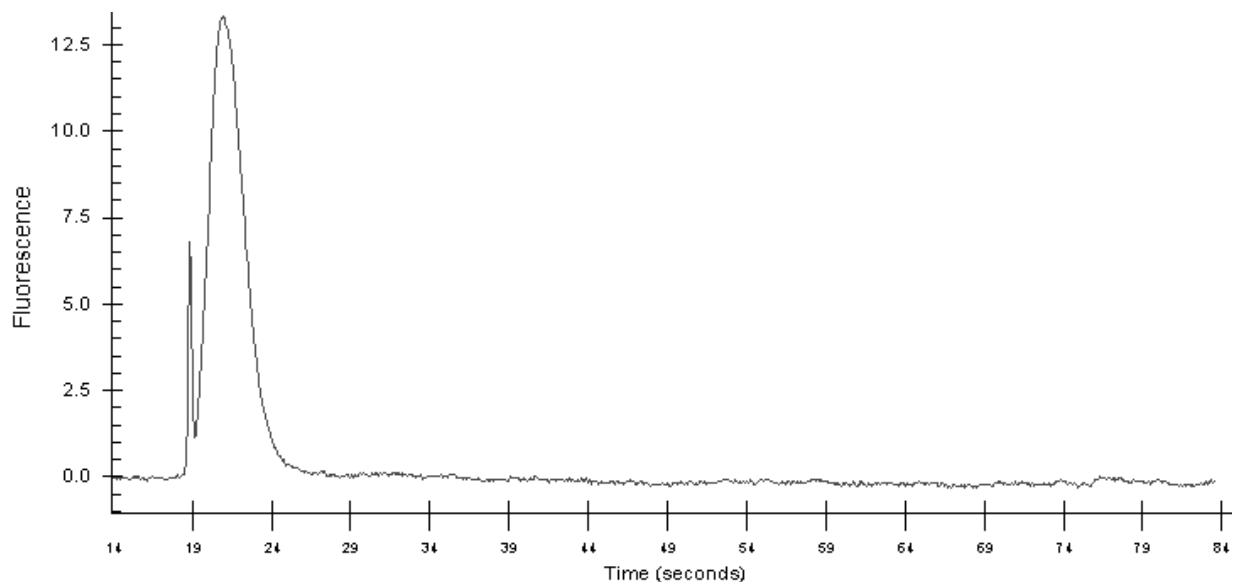


4.4 Fragmenting the cRNA for Target Preparation

1. The Fragmentation Buffer has been optimized to break down full-length cRNA to 35 to 200 base fragments by metal-induced hydrolysis. The following table shows the suggested fragmentation reaction mix for cRNA samples at a final concentration of 0.5 $\mu\text{g}/\mu\text{L}$.

Component	49 Format
cRNA	20ug(1-21 ul)
5XFragmentation Buffer	8ul
Rnase-free Water	bring to 40 ul final volume
total volume	40ul

- Incubate at 94°C for 35 minutes. Put on ice following the incubation.
- Save an aliquot (≥ 1 ul) for analysis on the Bioanalyzer. A typical fragmented target is shown :



Fragmented cRNA. Bioanalyzer electropherogram for fragmented labeled cRNA. This electropherogram displays the nucleotide size distribution for 150 ng of fragmented labeled cRNA resulting from one round of amplification. The average size is approximately 100 nt.

- Store undiluted, fragmented sample cRNA at -20°C (or -70°C for longer-term storage) until ready to perform the hybridization, as described in the *Eukaryotic Target Hybridization*

4.5 Eukaryotic Target Hybridization

- Mix the following for each target, scaling up volumes for hybridization to 21 arrays.

Component	Per Sample/49 Format (ul)	x 21 (ul)
Control Oligo B2 (3nM)	5.0	105.0
20X Eukaryotic Hybridization Controls (Bio B,C,D,Cre)	15.0	315.0
Herring Sperm DNA (10mg/ml)	3.0	63.0
BSA (50mg/ml)	3.0	63.0
2X Hybridization Buffer	150.0	3150.0
DMSO	30.0	630.0
Fragmented cRNA (15ug)	Variable	Variable
H2O	to Final Volume of 300	Variable
Total Volume	300.0	6300.0

It is imperative that frozen stocks of 20X GeneChip® Eukaryotic Hybridization Controls are heated to 65°C for 5 minutes to completely resuspend the cRNA before aliquotting.

2. Equilibrate probe array to room temperature immediately before use. *It is important to allow the arrays to equilibrate to room temperature completely. Specifically, if the rubber septa are not equilibrated to room temperature, they may be prone to cracking, which can lead to leaks.*
3. Heat the hybridization cocktail to 99°C for 5 minutes in a heat block.
4. Meanwhile, wet the array by filling it through one of the septa with appropriate volume of 1X Hybridization Buffer using a micropipettor and appropriate tips.
5. Incubate the probe array filled with 1X Hybridization Buffer at 45°C for 10 minutes with rotation.
6. Transfer the hybridization cocktail that has been heated at 99°C, in step 3, to a 45°C heat block for 5 minutes.
7. Spin hybridization cocktail(s) at maximum speed in a microcentrifuge for 5 minutes to remove any insoluble material from the hybridization mixture.
8. Remove the buffer solution from the probe array cartridge and fill with 200ul of the clarified hybridization cocktail, avoiding any insoluble matter at the bottom of the tube.
9. Place probe array into the Hybridization Oven, set to 45°C. Avoid stress to the motor; load probe arrays in a balanced configuration around the axis. Rotate at 60 rpm.
10. Hybridize for 16 hours.

4.6 *Washing, Staining, and Scanning*

Step 1: Defining File Locations

1. Launch Microarray Suite from the workstation and select **Tools** → **Defaults** → **File Locations** from the menu bar.
2. The File Locations window displays the locations of the following files:
 - a. Probe Information (library files, mask files)
 - b. Fluidics Protocols (fluidics station scripts)
 - c. Experiment Data (.exp, .dat, .cel, and .chp files are all saved to location selected here)
3. Verify that all three file locations are set correctly and click **OK**.

Step 2: Entering Experiment Information

To wash, stain, and scan a probe array, an experiment must first be registered in GCOS or Microarray Suite. Please follow the instructions detailed in the “*Setting Up an Experiment*” section of the appropriate GCOS or Microarray Suite User’s Guide. The fields of information required for registering experiments in Microarray Suite are:

- Experiment Name
- Probe Array Type

In GCOS, three additional fields are required:

- Sample Name
- Sample Type
- Project

Sample templates, Experiment templates, and array barcodes can also be employed in GCOS to standardize and simplify the registration process. Please see the GCOS User’s Guide for more information.

The Project, Sample Name, and Experiment Name fields establish a sample hierarchy that organizes GeneChip® gene expression data in GCOS. In terms of the organizational structure, the Project is at the top of the hierarchy, followed by Sample Name, and then Experiment Name.

For the MAQC study, use:

Project: **MAQC Main Study**

Sample Name: Platform_site_sample (e.g. **AFX_1_A**)

Experiment Name: Platform_site_sample replicate (e.g. **AFX_1_A1**)

Step 3: Preparing the Fluidics Station

The Fluidics Station 400, or 450/250 is used to wash and stain the probe arrays. It is operated using GCOS/Microarray Suite.

Setting Up the Fluidics Station

1. Turn on the Fluidics Station using the toggle switch on the lower left side of the machine.
2. Select **Run** → **Fluidics** from the menu bar. The Fluidics Station dialog box appears with a drop-down list for selecting the experiment name for each of the fluidics station modules. A second drop-down list is accessed for choosing the Protocol for each of the fluidics station modules.

Priming the Fluidics Station

Priming ensures that the lines of the fluidics station are filled with the appropriate buffers and the fluidics station is ready for running fluidics station protocols.

Priming should be done:

- when the fluidics station is first started.
 - when wash solutions are changed.
 - before washing, if a shutdown has been performed.
 - if the LCD window instructs the user to prime.
1. To prime the fluidics station, select Protocol in the Fluidics Station dialog box.
 2. Choose Prime or Prime_450 for the respective modules in the Protocol drop-down list.
 3. Change the intake buffer reservoir A to Non-Stringent Wash Buffer, and intake buffer reservoir B to Stringent Wash Buffer.
 4. In GCOS, select the All Modules check box, then click Run.
Refer to the Fluidics Station User's Guide for instructions on connecting and addressing multiple fluidics stations.

Step 4: Probe Array Wash and Stain:

After 16 hours of hybridization, remove the hybridization cocktail from the probe array and fill the probe array completely with the appropriate volume of Non-Stringent Wash Buffer(A).

(If necessary, at this point, the probe array can be stored at 4°C for up to 3 hours before proceeding with washing and staining. Equilibrate the probe array to room temperature before washing and staining.)

Preparing the Staining Reagents

SAPE Stain Solution

Streptavidin Phycoerythrin (SAPE) should be stored in the dark at 4°C, either foil-wrapped or kept in an amber tube. Remove SAPE from the refrigerator and tap the tube to mix well before preparing stain solution. Do not freeze SAPE. Always prepare the SAPE stain solution fresh, on the day of use.

Prepare the following reagents: SAPE Solution Mix for 1 and 3 stain and Antibody Solution Mix for stain 2.

SAPE Solution Mix

Component	Volume (ul)/Sample	X9 (ul)	X13 (ul)	X21 (ul)	Volume Final Concentration
2X Stain Buffer	600.0	5400.0	7800.0	12600.0	1X
50 mg/mL BSA	48.0	432.0	624.0	1008.0	2mg/ml
1 mg/mL Streptavidin Phycoerythrin (SAPE)	12.0	108.0	156.0	252.0	10µg/ml
DI H2O	540.0	4860.0	7020.0	11340.0	-
Total Volume	1200.0	10800.0	15600.0	25200.0	

Mix well and divide into two aliquots of 600 µL each to be used for stains 1 and 3.

Antibody Solution Mix

Component	Volume (ul)/Sample	X9 (ul)	X13 (ul)	X21 (ul)	Volume Final Concentration
2X Stain Buffer	300.0	2700.0	3900.0	6300.0	1X
50 mg/mL BSA	24.0	216.0	312.0	504.0	2mg/ml
10 mg/mL Goat IgG Stock	6.0	54.0	78.0	126.0	0.1mg/ml
0.5 mg/mL biotinylated antibody	3.6	32.4	46.8	75.6	3µg/ml
DI H2O	266.4	2397.6	3463.2	5594.4	-
Total Volume	600.0	5400.0	7800.0	12600.0	-

Mix well and divide into aliquots of 600 µL each to be used for stain 2.

All arrays will be run using **EukGE-ws2v5** fluidics protocol.

Step 5: Probe Array Scan

The scanner is also controlled by Affymetrix® Microarray Suite or GCOS. The probe array is scanned after the wash protocols are complete. Make sure the laser is warmed up prior to scanning by turning it on at least 15 minutes before use if you are using the Agilent GeneArray® Scanner, or 10 minutes if you are using the Affymetrix® GeneChip® Scanner 3000. If probe array was stored at 4°C, warm to room temperature before scanning. Refer to the Microarray Suite or GCOS online help and the appropriate scanner user's manual for more information on scanning.

1. On the back of the probe array cartridge, clean excess fluid from around septa.

2. Carefully apply one Tough-Spots to each of the two septa. Press to ensure that the spots remain flat. If the Tough-Spots do not apply smoothly, that is, if you observe bumps, bubbles, tears, or curled edges, do not attempt to smooth out the spot. Remove the spot and apply a new spot.
3. Insert the cartridge into the scanner and test the autofocus to ensure that the Tough-Spots do not interfere with the focus. If you observe a focus error message, remove the spot and apply a new spot. Ensure that the spots lie flat.

Scanning the Probe Array

1. Select **Run** → **Scanner** from the menu bar. Alternatively, click the **Start Scan** icon in the tool bar. The Scanner dialog box appears with a drop-down list of experiments that have not been run.
2. Select the experiment name that corresponds to the probe array to be scanned. A previously run experiment can also be selected by using the **Include Scanned Experiments** option box. After selecting this option, previously scanned experiments appear in the drop-down list.
3. By default, for the GeneArray® Scanner only, after selecting the experiment the number is displayed in the **Number of Scans** box to perform the recommended 2X image scan. For the GeneChip® Scanner 3000, only one scan is required.
4. Once the experiment has been selected, click the **Start** button. A dialog box prompts you to load an array into the scanner.
5. If you are using the GeneArray® Scanner, click the **Options** button to check for the correct pixel value and wavelength of the laser beam. Since you are using the GeneChip® Scanner 3000, pixel resolution and wavelength are preset and cannot be changed.
6. Open the sample door on the scanner and insert the probe array into the holder. Do not force the probe array into the holder. Close the sample door of the scanner.
7. Click **OK** in the Start Scanner dialog box.
The scanner begins scanning the probe array and acquiring data. When **Scan in Progress** is selected from the **View** menu, the probe array image appears on the screen as the scan progresses.

5. Data Management and Quality Control Specifications

- 5.1 One CAB file will be generated for this project with the project name as the title of the .CAB file
- 5.2 The quality report will be generated using Quality Reporter v1.0.04 for all arrays (<http://www.affymetrix.com/support/developer/tools/affytools.affx>)
 - 5.2.1 QC metrics should lie within 2 standard deviations of one another across the entire set of 20 arrays run; parameters such as scaling factor (SF with TGT200), percent present call, average background etc.
 - 5.2.2 P% >=45% and G3PDH 3'/5'<=2.0
 - 5.2.3 Minimum peak fragment size of cRNA 1000nt
 - 5.2.4 Peak size of fragmented cRNA ~100nt (50-200nt)